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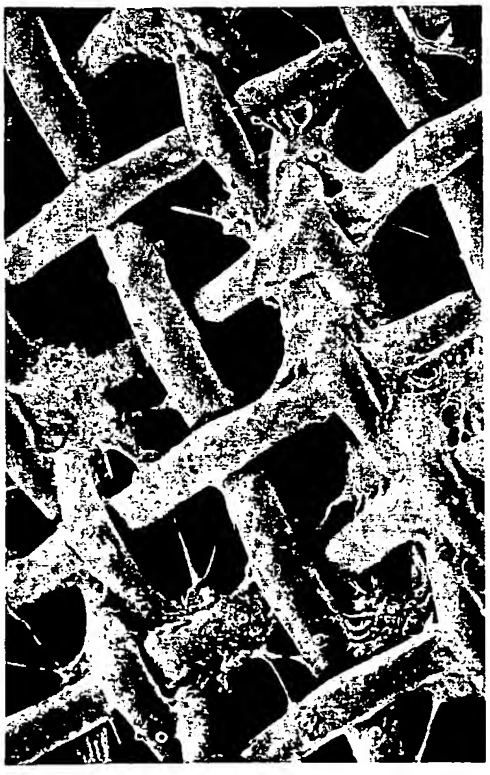

(57) Abstract: The present invention discloses compositions comprising a naturally secreted human extracellular matrix and methods for the use thereof. More particularly, the present invention provides compositions and methods for the repair of skin defects using natural human extracellular matrix by injection. The present invention also provides prosthetic devices which are coated or sealed with a composition comprising a naturally secreted extracellular matrix and methods for the use thereof.

WO 01/03750 A1

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# HUMAN NATURALLY SECRETED EXTRACELLULAR MATRIX-COATED DEVICE

This application is a continuation-in-part application of United States Patent Application Serial No. 08/660,787 filed June 6, 1996, which is a continuation-in-part of United States Patent Application Serial No. 08/470,101 filed June 6, 1995, now U.S. Patent No. 5,830,708, each of which is incorporated by reference herein in its entirety.

## 1. INTRODUCTION

The present invention is directed to a human naturally secreted extracellular matrix composition, as well as methods for the production and use thereof. In particular, the present invention is directed to methods of soft tissue repair by injecting a formulated human naturally secreted extracellular matrix composition into a subject in need thereof. The present invention is also directed to a formulated human naturally secreted extracellular matrix composition-coated prosthetic device for implantation into a subject, preferably a human, and more particularly to a prosthetic device such as a vascular graft or stent coated with a formulated human naturally secreted extracellular matrix composition of the present invention.

## 2. BACKGROUND OF THE INVENTION

The idea of using an injectable material for soft tissue augmentation and repair developed soon after the invention of the hypodermic needle. Various products have been injected into the human body for correction of soft tissue and skin defects including paraffin, petrolatum, vegetable oils, lanolin, bees wax, and silicone. Injectable liquid silicone has been used extensively, however, due to

long term side effects, such as nodules, recurring cellulitis and skin ulcers which are now being followed more closely, the use of injectable silicone is on the decline. Further, in the State of Nevada it is a felony to use injectable silicone in a human. Orange, Skin and Allergy News (1992) Vol.23, No.6, pg. 1. More recently, bovine collagen has gained widespread use as an injectable material for soft tissue augmentation. Collagen is the principal extracellular structural protein of the animal body. At least fourteen types of mammalian collagen have been described. The common characteristic amongst them is a three stranded helix, consisting of three polypeptide chains, called alpha-chains. All alpha-chains have the same configuration, but differ in the composition and sequence of their amino acids. Although this leads to different types of alpha-chains, however, they all have glycine at every third position in the amino acid sequence. The glycine at every third position allows for the helical structure of the alpha-chains. Type I collagen is composed of two $alpha_1$-chains and one $alpha_2$-chain and is the principal extracellular material of skin, tendon and bone. When clinicians mention "collagen", they are usually referring to type I collagen. See Table I, *infra*, for a detailed listing of collagen types I-V and in which tissues they are found.

Collagen has been used as an implant material to replace or augment hard or soft connective tissue, such as skin, tendon, cartilage, bone and interstitium. Additionally, collagen implants have been used for cosmetic purposes for a number of years since collagen can help cellular ingrowth at the placement site. Early collagen implants were often solid collagen masses which were cross-linked with chemical agents, radiation or other means to improve mechanical properties, decrease immunogenicity and/or increase resistance to resorption. The collagen utilized was

in a variety of forms, including cross-linked and non-cross-linked fibrillar collagens, gelatins, and the like and sometimes was combined with various other components, such as lubricants, osteogenic factors and the like, depending on use. A major disadvantage of solid cross-linked collagen implants is the requirement for surgical implantation by means of incision. In addition, lack of deformability and flexibility are other disadvantages of solid collagen implants.

Oliver et al., Clinical Orthopaedics & Related Research (1976) 115:291-302; Br. J. Exp. Path. (1980) 61:544-549; and Conn. Tissue Res. (1981) 9:59-62 describe implants made by treating skin with trypsin followed by cross-linking with an aldehyde. The resulting solid collagen implants were reported to maintain their original mass after prolonged implantation. A main problem with such solid implants is that they must be implanted surgically. Other disadvantages are that they are not as deformable as injectable implants and residual glutaraldehyde may cause the implant to lose its flexibility due to continuing cross-linking in situ.

Schechter, et al., Br. J. Plas. Surg. (1975) 28:198-202 disclose glutaraldehyde cross-linked skin that was soaked in L-alanine after cross-linking. The article postulates that the exposure of the skin to L-alanine blocked residual reactive groups of the aldehyde, thereby preventing the release of toxic molecules generated by such groups.

An alternative to surgically implanted solid collagen material is disclosed in U.S. Pat. No. 3,949,073. U.S. Patent No. 3,949,073 describes the use of atelopeptide solutions of bovine collagen as an injectable implant material for augmenting soft tissue. According to the patent, the bovine collagen is reconstituted before implantation and forms a fibrous mass of tissue when

implanted. The patent suggests adding particles of insoluble bovine collagen microfibrils to control the shrinkage of the fibrous mass formed at the augmentation site. The commercial embodiment of the material described in the patent is composed of reconstituted atelopeptide bovine collagen in saline that contains a small amount of local anesthetic. While effective, the implant shrinks in volume after implantation due primarily to absorption of its fluid component by the body. Thus, if volume consistency is essential, an additional injection or injections of supplemental implant material is required. This specific composition has many serious drawbacks, *e.g.*, the collagen is from a bovine source, not human, and the preparation process is not only lengthy and expensive but also requires the addition of microfibrils.

U.S. Patent No. 4,424,208 describes an injectable dispersion of cross-linked atelopeptide bovine collagen and reconstituted atelopeptide bovine collagen fibers in an aqueous carrier which exhibited improved volume consistency over the material of U.S. Pat. No. 3,949,073.

U.S. Patent No. 4,582,640 discloses an improved injectable implant over U.S. Pat. Nos. 3,949,073 and 4,424,208 in which the improvement consists of improved volume consistency and resistance to physical deformation, improved injectability as compared to the dispersion of U.S. Pat. No. 4,424,208 and that the bovine collagen contains only a single physical form of collagen as compared to the two physical forms found in U.S. Pat. No. 4,424,208.

U.S. Patent No. 4,803,075 describes bovine collagen compositions including a lubricant material to enhance injectability through narrow diameter needles for soft tissue repair.

Despite the advantages and overall usefulness of the injectable collagen implant materials disclosed above,

problems associated with producing and injecting the materials have been encountered. For example, for soft tissue repair, suspensions of fibrillar collagen have often been used by injecting the composition to a treatment site through a fine gauge needle. The use of fibrillar collagen as the primary matrix material in injectable soft and hard tissue implant compositions has several limitations. The preparation of fibrillar collagen suitable for human use is relatively time consuming and expensive. In particular, the complete removal of contaminating and potentially immunogenic substances to produce atelocollagen is a relatively complex and expensive procedure. Moreover, the persistence, shape retention, cohesiveness, stability, elasticity, toughness and intrudability of the fibrillar collagen compositions are not optimal.

In addition to the problems associated with producing and injecting the collagen implant materials, problems with the actual use of the above mentioned patented injectable implants are also abundant. For instance, since the above patented injectables derive collagen from xenogeneic sources, usually bovine collagen, the collagen must be modified to reduce its immunogenicity. Even with modified collagen, the implant material is still quite immunogenic to which some people are either already naturally allergic or develop an allergic reaction over time to the bovine collagen. Due to these allergic reactions the injectable collagen implants described above cannot be given to many people and others are limited to receiving only three injections per year. Severe allergic reactions include symptoms of rheumatoid arthritis, while less severe reactions include redness and swelling at the site of injection which may lead to permanent scarring. Because of these severe side effects, the above described collagen injectables are no longer used for lip augmentation. Further, the problems

associated with injecting xenogeneic collagen seem so intractable that rather than injecting collagen, biocompatible ceramic matrices have been injected to achieve similar results as described in U.S. Patent No. 5,204,382.

In summary, due to the shortcomings of the above-described injectable compositions for the repair of soft tissue defects, such as the lack of persistence, the need for repeated injections and serious concern over adverse reactions, newer injectable materials for soft tissue augmentation are needed.

## 3. SUMMARY OF THE INVENTION

The present invention relates to injectable materials for soft tissue augmentation and methods for use and manufacture of the same, which overcome the shortcomings of bovine injectable collagen and other injectable materials, including silicone, of the prior art. The injectable materials used in accordance with the present invention comprise naturally secreted extracellular matrix preparations as well as preparations derived from naturally secreted extracellular matrix. These preparations are biocompatible, biodegradable and are capable of promoting connective tissue deposition, angiogenesis, reepithelialization and fibroplasia, which is useful in the repair of skin and other tissue defects. These extracellular matrix preparations may be used to repair tissue defects by injection at the site of the defect.

The injectable preparations of the present invention have many advantages over conventional injectable collagen preparations used for the repair of skin defects. The extracellular matrix preparations of the present invention contain only human proteins, therefore, there is a reduced risk of an immune response due to foreign, *e.g.*, xenogeneic, proteins or peptides, especially the type of

immune response seen with bovine collagen found in conventional injectable collagen preparations. Additionally, the injected preparations of the present invention should persist longer, and, even if multiple injections are required, the injections should not be subject to the "no more than three injections per year" rule of bovine collagen-based preparations due to the lack of immunogenicity. Another advantage provided by the present invention is that the preparations of naturally secreted extracellular matrix contain a mixture of extracellular matrix proteins that closely mimics the compositions under physiologically normal conditions; for example, in an extracellular matrix derived from dermal cells, type I and III collagens, hyaluronic acid as well as various glycosaminoglycans and natural growth factors are present. Many of these extracellular matrix proteins and growth factors have been studied extensively and have been shown to be critical for wound healing and tissue restoration.

The present invention also relates to a prosthetic device suitable for use or implantation into a subject, preferably a human. The device is coated with a formulated human naturally secreted extracellular matrix composition. The device, for example, is a annuloplasty ring, heart sewing ring, stent, artificial joint, artificial heart, etc. In another embodiment, the device is a suture material, gauze pad or an adhesive or non-adhesive bandage. The formulated materials used in accordance with the present invention comprise human naturally secreted extracellular matrix preparations as well as preparations derived from human naturally secreted extracellular matrix. These compositions are biocompatible, biodegradable and are capable of promoting connective tissue deposition, angiogenesis, reepithelialization and fibroplasia, which is useful in the promotion of wound healing and tissue regeneration. The

present invention also provides new and advantageous processes for generating the extracellular matrix coated devices suitable for implantation.

The devices of the present invention have many advantages over conventional devices used for wound repair or in surgery. The extracellular matrix preparations of the present invention which coat or seal the device contain only human proteins, therefore, there is a reduced risk of an immune response due to foreign, *e.g.*, xenogeneic, proteins or peptides. Another advantage provided by the present invention is that the preparations of naturally secreted extracellular matrix contain a mixture of extracellular matrix proteins which closely mimics the compositions under physiologically normal conditions, for example, in an extracellular matrix derived from dermal cells, type I and III collagens, hyaluronic acid as well as various glycosaminoglycans and natural growth factors are present. Many of these extracellular matrix proteins and growth factors have been studied extensively and have been shown to be critical for wound healing and tissue restoration and regeneration. In a specific embodiment, the extracellular matrix is formulated with a drug, *e.g.*, an antibiotic, angiogenesis factor, other therapeutic agent., such that the implantable device coated with the formulated matrix also acts as a drug delivery system. In one embodiment of the present invention, the composition is an autologous composition prepared according to the methods of the invention using cells or tissues obtained from the subject in which the device is to be implanted.

In another embodiment of the invention, the matrix preparations can be used in highly improved systems of *in vitro* tissue culture. In this embodiment, naturally secreted extracellular matrix coated three-dimensional frameworks can be used to culture cells, which require attachment to a

support in order to grow, but do not attach to conventional tissue culture vessels. In addition to culturing cells on a coated framework, the extracellular matrix secreted by the cells onto the framework can be collected and used to coat vessels used in tissue culture. The extracellular matrix, acting as a base substrate, may allow cells normally unable to attach to conventional tissue culture dish base substrates to attach and subsequently grow.

Yet another embodiment of the present invention is directed to a novel method for determining the ability for cellular taxis of a particular cell. The method involves inoculating one end of a naturally secreted extracellular matrix coated three-dimensional framework with the cell type in question and over time measure the distance traversed across the framework by the cell. Because the extracellular matrix is secreted naturally by the cells onto the framework, it is an excellent *in vitro* substitute of extracellular matrix found in the body. Such an assay, for example, may inform whether isolated tumor cells are metastatic or whether certain immune cells can migrate, or even chemotact, across the framework, thus, indicating that the cell has such cellular taxis ability.

### 3.1. DEFINITIONS AND ABBREVIATIONS

The following terms used herein shall have the meanings indicated:

Adherent Layer:

cells attached directly to the three-dimensional framework or connected indirectly by attachment to cells that are themselves attached directly to the matrix.

Naturally Secreted:

in context of a naturally secreted three-dimensional extracellular matrix, naturally

secreted means that the extracellular matrix is secreted by cells growing in three dimensions as opposed to cells growing in monolayer culture, such that the matrix composition secreted by the cells more closely resembles the matrix as secreted by cells *in vivo*.

Pharmaceutically Acceptable Carrier:

an aqueous medium at physiological isotonicity and pH and may contain other elements such as local anesthetics and/or fluid lubricants.

Stromal Cells:

fibroblasts with or without other cells and/or elements found in loose connective tissue, including but not limited to, endothelial cells, pericytes, macrophages, monocytes, plasma cells, mast cells, adipocytes, chondrocytes, etc.

Three-Dimensional Framework:

a three dimensional support composed of any material and/or shape that (a) allows cells to attach to it (or can be modified to allow cells to attach to it); and (b) allows cells to grow in more than one layer. This support is inoculated with stromal cells to form the living stromal matrix.

Living Stromal Tissue:

a three dimensional framework which has been inoculated with stromal cells. Whether confluent or subconfluent, stromal cells according to the invention continue to grow and divide. The living stromal tissue prepared *in vitro* is the source of the extracellular matrix proteins used in the injectable formulations of the invention.

The following abbreviations shall have the meanings indicated:

| | |
|---|---|
| EDTA | ethylene diamine tetraacetic acid |
| FBS | fetal bovine serum |
| HBSS | Hank's balanced salt solution |
| HS | horse serum |
| MEM | minimal essential medium |
| PBS | phosphate buffered saline |
| RPMI 1640 | Roswell Park Memorial Institute Medium No. 1640 (GIBCO, Inc., Grand Island, NY) |
| SEM | scanning electron microscopy |

The present invention may be more fully understood by reference to the following detailed description, examples of specific embodiments and appended figures, which are offered for purposes of illustration only and not by way of limitation.

## 4. BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1. Figure 1 is a scanning electron micrograph depicting fibroblast attachment to the three-dimensional matrix and extension of cellular processes across the mesh opening. Fibroblasts are actively secreting matrix proteins and are at the appropriate stage of subconfluency which should be obtained prior to inoculation with tissue-specific cells.

FIGURE 2A-D. Figures 2A-D are transmission electron micrographs of collagen isolated from extracellular matrix prepared from dermal tissue grown *in vitro* (Figure 2A-B) or from a normal adult human dermal sample (Figure 2C-D).

## 5. DETAILED DESCRIPTION OF THE INVENTION

One embodiment of the present invention involves the preparation and use of an injectable extracellular matrix composition for the treatment of skin defects. The extracellular matrix proteins are derived from a living stromal tissue prepared *in vitro* by growing stromal cells on

a three-dimensional framework resulting in a multi-layer cell culture system. In conventional tissue culture systems, cells are grown in a monolayer. Cells grown on a three-dimensional framework support, in accord with the present invention, grow in multiple layers, forming a cellular matrix. This matrix system approaches physiologic conditions found *in vivo* to a greater degree than previously described monolayer tissue culture systems. The three-dimensional cell culture system is applicable to the proliferation of different types of stromal cells and formation of a number of different stromal tissues, including but not limited to, dermis, bone marrow stroma, glial tissue, cartilage, etc.

In accordance with the present invention, the pre-established living stromal tissue comprises stromal cells grown on a three-dimensional framework or network. The stromal cells can comprise fibroblasts with or without additional cells and/or elements described more fully herein. The fibroblasts and other cells and/or elements that comprise the stroma can be fetal or adult in origin, and can be derived from convenient sources such as skin, liver, pancreas, etc. Such tissues and/or organs can be obtained by appropriate biopsy or upon autopsy. In fact, cadaver organs may be used to provide a generous supply of stromal cells and elements.

Once inoculated onto the three-dimensional framework, the stromal cells will proliferate on the framework, and elaborate growth factors, regulatory factors and extracellular matrix proteins that are deposited on the support. The living stromal tissue will sustain active proliferation of the culture for long periods of time. Growth and regulatory factors can be added to the culture, but are not necessary since they are elaborated by the stromal support matrix. Methods and apparatus for preparing

such living three-dimensional stromal tissues and resulting compositions, such as vascular graft compositions, can be found in United States Patent Nos. 4,963,489; 5,266,480; and 5,792,603, each of which is incorporated by reference herein in its entirety.

The naturally secreted extracellular matrix is collected from the three-dimensional framework and is processed further with a pharmaceutically acceptable carrier and placed in a syringe for precise placement of the biomaterial into tissues, such as the facial dermis, or into the sac of an aneurysm. The injectable compositions are also useful for the repair of spinal and craniofacial defects.

In another embodiment of the present invention, the naturally secreted extracellular matrix is collected from the three-dimensional framework and is processed further for coating or sealing a prosthetic device which is suitable, *e.g.*, for implantation into a subject, preferably a human. Examples of such devices include, but are not limited to, a stent, graft, stent/graft, synthetic graft, tissue engineered vascular graft, as well as sewing and annuloplasty rings used in cardiac valve reconstruction and replacement. Further, such devices may be constructed from metal or plastic or biopolymers. In yet another embodiment, the extracellular matrix is used to coat or seal internal or external surgical sutures, as well as for coating Band-Aid®-type adhesive bandages and other wound healing coverings, *e.g.*, non-adhesive gauze.

The present invention is based, in part, on the discovery that during the growth of human stromal cells on a biodegradable or non-biodegradable three-dimensional support framework, the cells synthesize and deposit on the three-dimensional support framework a human extracellular matrix as produced in normal human tissue. The extracellular matrix is secreted locally by cells and not only binds cells

and tissues together but also influences the development and behavior of the cells it contacts. The extracellular matrix contains various fiber-forming proteins interwoven in a hydrated gel composed of a network of glycosaminoglycan chains. The glycosaminoglycans are a heterogeneous group of long, negatively charged polysaccharide chains, which (except for hyaluronic acid) are covalently linked to protein to form proteoglycan molecules.

The fiber-forming proteins are of two functional types: (a) mainly structural (collagens and elastin), and (b) mainly adhesive (such as fibronectin and laminin). The fibrillar collagens (types I, II, and III) are rope-like, triple-stranded helical molecules that aggregate into long cable-like fibrils in the extracellular space; these in turn can assemble into a variety of highly ordered arrays. Type IV collagen molecules assemble into a sheetlike meshwork that forms the core of all basal laminae. Elastin molecules form an extensive cross-linked network of fibers and sheets that can stretch and recoil, imparting elasticity to the matrix. Fibronectin and laminin are examples of large adhesive glycoproteins in the matrix; fibronectin is widely distributed in connective tissues, whereas laminin is found mainly in basal laminae. By means of their multiple binding domains, such proteins help cells adhere to and become organized by the extracellular matrix.

As an example, a naturally secreted human dermal extracellular matrix contains type I and type III collagens, fibronectin, tenascin, glycosaminoglycans, acidic and basic FGF, TGF-α and TGF-β, KGF, decorin and various other secreted human dermal matrix proteins. As naturally secreted products, the various extracellular matrix proteins are produced in the quantities and ratios similar to that existing *in vivo*. Moreover, growth of the stromal cells in three dimensions will sustain active proliferation of cells